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INTRODUCING A BETA-AMYLASE GENE WITH HIGH THERMOSTABILITY AND LOW INHIBITORS-BINDING ACTIVITY INTO CANADIAN MALTING BARLEY USING MOLECULAR TECHNIQUES

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Introducing a *beta*-amylase gene with high thermostability and low inhibitor-binding activity into Canadian malting-barley using molecular techniques.

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Final Report

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Peter Eckstein, Eric B. Lefol, Brian G. Rossnagel, Bryan L. Harvey, and Graham J. Scoles
Department of Plant Sciences
University of Saskatchewan
51 Campus Drive
Saskatoon, Saskatchewan
S7N 5A8

ABSTRACT

Barley *beta*-amylase thermostability and inhibitor-binding capacity are important factors in malting, and are determined by a series of single nucleotide substitutions within the gene *Bmy*1, on chromosome 4H of barley. A series of molecular markers have been developed to characterize these substitutions and to enable marker-assisted selection for the trait. The thermostable *beta*-amylase has been introgressed into Canadian cultivars/lines through a series of crosses with a Japanese thermostable cultivar, and the aid of the markers. Examinations of genetic diversity at *Bmy*1 suggests that the thermostable variant may already be present, and may be used to improve malting quality, in western Canadian cultivars.

EXECUTIVE SUMMARY

Barley is the major raw material for the malting and brewing industry. The process of malting changes the starch found in the barley grain into fermentable sugars. The major enzymes involved in this process are *alpha*-amylase, *beta*-amylase, limit dextrinase and *alpha*-glucosidase and their combined activity is called diastatic power (DP) which directly affects malt yield and quality. The brewing industry prefers barley with high DP resulting in a high yield of sugars, shorter mashing times, and the ability to add more adjunct (other sources of starch). *Beta*-amylases are the most important enzymes involved in the conversion of starch stored in the barley grain to simple sugars, and are fundamental in determining the DP of malting-barley. This enzyme may also combine with an inhibitor known as protein Z which influences the quality and stability of beer. Several factors determine the enzyme efficiency during malting and brewing including total enzyme activity, the level of enzyme inhibitor, the degree to which the enzyme binds to the inhibitor, and the thermostability of the enzymes during kilning and mashing (during which enzyme activity is lost due to the relatively high temperatures used). *Beta*-amylases with high thermostability and low inhibitor-binding activity provide high levels of DP as well as low levels of protein, both desired by the malting industry. Canadian malting-barley cultivars, of which Harrington is an example, have *beta*-amylases (Sd1) with low thermostability and high inhibitor-binding activity (30% more) compared with the *beta*-amylase (Sd2) in Japanese malting-barley cultivars such as Haruna Nijo. An improvement in the thermostability and a reduction of the inhibitor-binding activity of the *beta*-amylase present in Canadian cultivars would improve the malting quality of Canadian grown barley.

In order to increase the DP while maintaining the low protein characteristic of Canadian malting-barley, the gene responsible for the Sd2 type *beta*-amylase was introduced into Canadian barley germplasm. Crosses between the Japanese Sd2 type cultivar Haruno Nijo and four cultivars/lines from the Crop Development Centre barley breeding program were made and advanced through the breeding program. Populations were generated through conventional single-seed-descent breeding methods, as well as protocols for doubled haploid production using microspore culture.

The *Bmy*1 gene of barley, located on chromosome 4H, that encodes different forms of *beta*-amylase, was isolated and sequenced. By comparing the amino acid sequence of the Sd1 and Sd2 variants of *Bmy*1, the underlying molecular differences leading to the high thermostability and low inhibitor-binding activity of Sd2 *beta*-amylase has become clearer.

An analysis of the DNA sequences, and translation to the amino acid sequence of both forms of the gene revealed several nucleic acid substitutions resulting in six specific amino acid substitutions. A specific combination of these substitutions is responsible for differences in both the thermostability and inhibitor-binding activity of the Sd1 and Sd2 forms. Furthermore, regions of DNA sequence located within an intron (non-coding) region of the gene are implicated in the determination of thermostability, confirming a previously published finding.

Based on the elucidated nucleotide sequence of *Bmy*1 and the variation that exists at the DNA level between the Sd1 and Sd2 forms of the gene, a series of molecular markers were designed. Nine markers were constructed of which six were dominant SCAR markers, two were co-dominant SCAR markers, and one was based on a microsatellite region.

Molecular markers have the advantage of testing directly for the presence of a certain gene sequence as opposed to selecting for the product the gene encodes. Using markers, DNA of the lines originating from crosses between Haruna Nijo and the Canadian lines/cultivars was screened for the presence or absence of the Sd2 form of *Bmy*1 (a process known as molecular marker assisted selection MMAS). This increased the accuracy of the selections, and reduced the cost and time involved for conventional determinations of *beta*-amylase thermostability and DP. Many of the markers were shown to be highly polymorphic and have the potential to select for the desired characteristic in a wide array of the germplasm. In addition, these molecular markers have been used to identify the form of the gene present in other cultivars that may be useful in future crosses. Several Canadian feed-barley lines carrying the Sd2 form of *beta*-amylase were identified. Of these, two sister lines (HB334 and HB345) have been sequenced in their entirety and compared to the sequence of Haruna Nijo to confirm that the forms of the gene are identical. The thermostable form of the enzyme therefore already exists in barley well-adapted to the growing conditions of western Canada.

We have demonstrated the efficient use of microspore culture for the rapid generation of breeding populations in barley. The analysis of the Sd2 type *beta*-amylase gene, the transfer of this form of the enzyme into western Canadian cultivars/lines, and the molecular marker accelerated selection of desirable lines has generated plant material that will form the basis for the improvement of malting quality. This will allow Canadian malting-barley cultivars to remain competitive with those of Australia and Japan in international markets.

In addition, the new insights into the nature of the differences between Sd1 and Sd2 forms of *Bmy*1 are an important contribution to the current knowledge of barley *beta*-amylase.

TECHNICAL REPORT

Objectives and Research Plan

The objectives of the proposed study were: 1) to isolate the *beta*-amylase gene (located on barley chromosome 4) from "Harrington" and Japanese malting-barley, and to understand the molecular mechanisms that determine the thermostability and inhibitor-binding activity of the *beta*-amylase gene in Japanese germplasm: 2) develop molecular markers that target the *beta*-amylase gene, and: 3) use the molecular markers to assist in transferring the Japanese heat-stable *beta*-amylase gene into Canadian malting-barley cultivars.
The following activities were included in our research plan;

1. Harrington and three other Canadian cultivars/advanced lines will be crossed with Haruna Nijo, and populations produced using microspore culture (doubled haploid (DH) lines) or single-seed-decent (recombinant-inbred-lines RIL).
2. Messenger RNA will be isolated from the developing seed of Harrington and Haruna Nijo.
3. The *beta*-amylase genes specifically expressed in the seed will be cloned and sequenced using RT-PCR.
4. The two *beta*-amylase sequences will be compared and differences between the two that relate to variations in thermostability and inhibitor binding activity will be identified.
5. SCAR markers will be developed that target the differences between the *beta*-amylase genes, and other markers linked to this locus will be developed based on comparative maps.
6. The segregating populations will be screened with AFLP markers (used to eliminate the genetic background of Haruna Nijo) and the *beta*-amylase specific markers.
7. The thermostability (DP) of the selected lines will be assessed (to verify the incorporation of the desirable Sd2 type *Bmy*1 gene into Canadian cultivars/lines).

The following section details our results according to the research plan. It should be noted that re-analysis of results and modifications to some of the molecular markers may have altered some information given in previous progress reports.

Plant Material

The Japanese malting-barley cultivar Haruna Nijo, which carries the desirable Sd2 thermostable form of the *beta*-amylase gene, was crossed with four Canadian Sd1 type malting-barley cultivars/lines: Harrington, TR253, SM94291 and CDC Kendall. Several crosses were made with each parent to guard against seed heterogeneity, or failed crosses. Seed of the actual parent lines/plants used in the crosses was retained for future molecular analysis. Of the original 16 crosses, three failed or were discarded. The remaining 13 populations (Table 1) were developed using microspore culture technology to quickly generate doubled haploid (DH) populations. Doubled haploid populations are completely homogeneous with no remaining heterozygosity after only a single generation. The advantage of the DH process is that the thermostable characteristic would be fixed (no longer segregating) and could be selected using molecular markers early in the breeding process.
Since our microspore culture technique was under development at the time, the populations were simultaneously advanced as RILs to guard against failure of the DH process.

Table 1. Crosses between Japanese cultivar Haruna Nijo, and four Canadian malting-barley cultivars/lines.

Cross#	Pedigree
68A	Haruna Nijo #1/RFLP Harrington #1
68C	Haruna Nijo #3/RFLP Harrington #1
68D	Haruna Nijo #5/RFLP Harrington #2
69A	Haruna Nijo #1/TR253 #2
69B	Haruna Nijo #2/TR253 #3
69C	Haruna Nijo #4/TR253 #3
69D	Haruna Nijo #6/TR253 #2
70A	Haruna Nijo #3/SM94291 #2
70C	Haruna Nijo #5/SM94291 #1
70E	Haruna Nijo #6/SM94291 #1
71A	Haruna Nijo #1/CDC Kendall #1
71B	Haruna Nijo #5/CDC Kendall #1
71C	Haruna Nijo #6/CDC Kendall #2

Isolation of *Bmy1* and DNA sequence analysis.

Plants of the barley cultivars Harrington and Haruna Nijo were grown in a growth chamber to a stage where the developing heads were clearly formed but had not yet emerged from the sheath (52 – 59 days post-seeding). Total RNA was extracted from the developing heads of the plants using a RNeasy® extraction kit (Qiagen) according to manufacturer's instructions. The *Bmy*1 gene from both barley cultivars was isolated from the total RNA sample through reverse transcription PCR (RT-PCR). The PCR primers for RT-PCR were based on DNA sequence of the *Bmy*1 gene that had previously been isolated and sequenced (Kreis et al., 1987). The *Bmy*1 gene (cDNA) was cloned into the plasmid pCR2.1 (Invitrogen) and sequenced at the DNA sequencing facility at the Plant Biotechnology Institute (Sakatoon).

The DNA sequence of the isolated Haruna Nijo *Bmy*1 cDNA was compared to DNA sequence of the *beta*-amylase gene from Haruna Nijo (Genbank accession #D49999). Both sequences were a perfect match, confirming that the correct gene had been isolated. The DNA sequence of *Bmy*1 from Harrington (Sd1) was then compared to *Bmy*1 sequences from Sd2 type cultivars Haruna Nijo, Adorra (Genbank Accession #AF061203), Hiproly (#X52321), and Galleon. Sequence comparisons revealed several nucleotide substitutions, of which seven substitutions resulted in amino acid (aa) substitutions between the five cultivars. Four aa substitutions were evident between Haruna Nijo and Harrington, with three more between Haruna Nijo and the other cultivars. Six of the seven nucleotide and aa substitutions are listed in Table 2. The first substitution, which occurred only in the Australian variety Galleon at aa position 85 was not included in this study. The complete aa sequence comparison of all 5 cultivars is shown in Appendix A.

Table 2. Nucleotide and amino acid substitutions (with position) within *Bmy*1.

Substitution	Nucleotide (position)	Amino Acid (position)
1	Cytosine/Thymine 343	Arg/Cys 115
2	Cytosine/Guanine 495	Asp/Glu 165
3	Cytosine/Thymine 698	Ala/Val 233
4	Cytosine/Thymine 1040	Ser/Leu 347
5	Thymine/Cytosine 1289	Val/Ala 430
6	Guanine/Adenine 1581	Met/Ile 527

Development of molecular markers

To use molecular markers to select for a particular characteristic, they must show a "polymorphism", or difference in banding pattern, that can be associated with the presence or absence of a particular allele. To be assured enough polymorphism existed so that any of the populations could be screened, numerous markers where developed. In addition, numerous markers were required to effectively study the variation within the *Bmy*1 sequence, and to establish the molecular basis for thermostability.

Sequence characterized amplified region (SCAR) markers were developed to target each of the six single base changes that resulted in amino acid substitutions. For each SCAR marker, a set of two PCR primers was designed to selectively amplify one of the two alleles at each locus, and to produce a single band only if a specific allele was present. The primers for each of the six SCAR markers are listed in Table 3. The PCR products of each of the six markers, and the allele that is associated with the positive signal, are shown in Appendix B.

Two additional markers were produced, based on previously published work, to further evaluate the genetic diversity at the *Bmy*1 locus;

a. A SCAR marker (BmyIntron3) was designed to amplify a region of DNA containing a 126bp palindromic insertion within intron III of the genomic *beta*-amylase sequence. This region has previously been implicated as playing a role in thermostability (Erkilla et al., 1998). This marker is "co-dominant" and produces a single band of different size for either allele.
b. PCR primers (BmyMicros) were designed to amplify a microsatellite ($TG_{10}G_{16}$) containing region of DNA at nucleotide1849 within intron III of the genomic sequence of the *beta*-amylase gene.

A further SCAR marker was produced using a set of PCR primers (Bmy21I/D) that amplify a region of DNA containing a 19bp insertion/deletion in intron III of the *beta*-amylase gene, which was discovered during the course of this research. This marker is also co-dominant. It should be noted that this development led to changes in PCR primers and primer names as compared to previous progress reports.

The primer sequences, amplified fragment lengths, and annealing temperatures of all markers are listed in Table 3. PCR reaction conditions were optimized for each of the markers such that each marker produces a consistently clear and easy to interpret result.

Assessment of marker polymorphism on parental materials

The eight SCAR markers were used to screen the DNA of the individual parents used in the crosses to establish the amount of available polymorphism and the results are listed in Table 4.

The banding patterns of all eight markers indicated that the individual parent plants within a cultivar (eg Haruna Nijo 1-6) used in the crosses were identical. These results confirm that the parental seed samples of all the cultivars used in the crosses were genetically uniform.

Six of the eight SCAR markers (BmyR115, BmyE165, BmyV233b, BmyS347b, BmyA430, Bmy21I/D) were polymorphic between Haruna Nijo and all four other parents, indicating that any of those markers could be used to select for the desirable thermostable Sd2 form of *beta*-amylase from Haruna Nijo. The high amount of polymorphism was beneficial in that;

a. the final selection of crosses to be screened using markers and evaluated for enzyme thermostability, could be based on population quality and size.
b. more than one marker could be used as a selection tool in the event that PCR reaction conditions changed and a marker became less useful.
c. screening results from one marker could be confirmed by results from a second marker. This is especially important when using dominant markers (such as SCARs) in which the marker test results are a single band versus no band at all, and a negative result may be the result of a failed PCR reaction.
d. since the markers are spaced throughout the gene, a marker located near the beginning of the gene (BmyR115) may be used with a marker located near the other end of the gene (BmyA430) to confirm that a large part (if not the entire) gene is present.

Furthermore, the large amount of polymorphism implies that the markers will likely be applicable in many future crosses that will be made to continuously incorporate the Sd2 *beta*-amylase.

Markers BmyM527 and BmyIntron3 were not polymorphic in the crosses created for this project and could therefore not be used for marker-assisted selection.

Markers based on microsatellite DNA are useful because they are more often polymorphic between the parents of a particular cross than SCAR markers. The microsatellite marker (BmyMicros) was highly polymorphic among the parents used in the crosses. However, since screening with microsatellite markers involves the use of polyacrylamide gels and more technical expertise and time, and since many of the SCAR markers worked effectively and were highly polymorphic, the microsatellite marker was not used to select for the thermostable *beta*-amylase.

Table 3. PCR primers, length of amplified DNA fragment, and optimal annealing temperature (Tm) for nine molecular markers for *Bmy*1 in barley.

Marker Name	Nucleotide Sequence	Fragment Length (bp)	Annealing Temperature (°C)
BmyR115	GCC GTG AGC GTG AAC AAC AG	306	68.5
	GTA GAA AAT GTC GGG ATC ACG		
BmyE165	AAG GCT GGG CTG AAG CTA	654	68
	CGA TAA CAC CAG CAT CCA AGA AC		
BmyV233B	CAG ACA GTT CAC CCA TAA GCT	482	61
	GTG TCA TTG TAC TGT CCG G		
BmyS347B	CAG ACA GTT CAC CCA TAA GCT	1023	62
	CCT GCG AGC TTT GCT CCG		
BmyA430	GGT GGA GGG ACA AAA CTA TGC	508	69
	GGT TTC TCT GTC ACA CTC ACA		
BmyM527	CAG GGA GCT CCC CAC CC	451	67
	AGC TGG TGG AGG GAC AAA		
BmyIntron3	CAG GCA TCG GAG AAT TCA	471/597	58
	AGA GAC TAC AAT GTG CCG AA		
BmyMicros	TTA GCA CAA ACC TTG ATG CAA G	352	56.5
	TGC TGC TGC TTT GAA GTC TGC		
Bmy21I/D	TGC TGC TGC TTT GAA GTC TGC	400/419	56.5
	CAG ACA GTT CAC CCA TAA GCT		

Table 4. Banding pattern results for 8 SCAR markers on DNA of all parental materials used in crosses.

Parent	Bmy R115	Bmy E165	Bmy V233B	Bmy S347B	Bmy A430	Bmy M527	Bmy 21I/D	Bmy Intron3
Haruna Nijo #1	+	-	+	+	-	+	long	short
Haruna Nijo #2	+	-	+	+	-	+	long	short
Haruna Nijo #3	+	-	+	+	-	+	long	short
Haruna Nijo #4	+	-	+	+	-	+	long	short
Haruna Nijo #5	+	-	+	+	-	+	long	short
Haruna Nijo #6	+	-	+	+	-	+	long	short
Harrington #1	-	+	-	-	+	+	short	short
Harrington #2	-	+	-	-	+	+	short	short
TR253 #2	-	+	-	-	+	+	short	short
TR253 #3	-	+	-	-	+	+	short	short
SM94291 #1	-	+	-	-	+	+	short	short
SM94291 #2	-	+	-	-	+	+	short	short
CDC Kendall #1	-	+	-	-	+	+	short	short
CDC Kendall #2	NT	NT	NT	NT	NT	NT	NT	NT

Selection of crosses for molecular marker-assisted selection

Doubled haploids – Of the 13 crosses subjected to microspore culture for the production of doubled haploids, four populations (one population using each of the four Canadian parents) were retained and propagated in the greenhouse for seed increase. These crosses were 68C, 69B, 70E, and 71B. The crosses were chosen on the basis of population size. Two populations were selected in which progeny carrying the normal and the thermostable forms of *beta*-amylase could be identified through the use of markers. These crosses were chosen on the basis of population size, seed availability, and the greatest amount of variation in DP between the parents of the cross. The crosses were Haruna Nijo x TR253 (MC69B) and Haruna Nijo x CDC Kendall (MC71B).

Recombinant Inbred Lines - All 13 crosses were advanced through successive greenhouse and field generations to F_6 populations. These populations were not chosen for marker-assisted selection since the DH populations were successfully produced. Some of the RIL populations were field evaluated for agronomic characteristics and remain in storage for possible future evaluation.

Marker-assisted selection

Cross MC69B – progeny of the cross were screened using three SCAR markers (BmyE165, BmyS347B, BmyA430). Of the 127 total DH lines, 11 failed to germinate and three lines failed to consistently produce a PCR product. These lines were eliminated from further analysis. Of the remaining 113 lines, 46 carried the Haruna Nijo Sd2 *beta*-amylase allele and 67 carried the Sd1 *beta*-amylase allele.

Cross MC71B – progeny of the cross were screened using SCAR markers BmyS347b and BmyA430. Of the 81 DH lines, 32 carried the Haruna Nijo Sd2 allele while 49 carried the Sd1 allele.

In both crosses, the number of lines carrying the Haruna Nijo Sd2 allele was lower than the number carrying the Sd1 allele. This can be attributed to the fact that the DH populations were field-grown to increase the amount of available seed for field trials, and that the final lines to be evaluated were chosen based on seed quality and amount. It is conceivable that lines carrying large amounts of Haruna Nijo DNA were not as adapted to western Canadian growing conditions and were therefore selected against. In future, it may be advantageous to introgress the thermostable *beta*-amylase from Japanese germplasm using a backcrossing scheme which minimizes the amount of "donor" (in this case Japanese) DNA.

AFLP genotyping

Amplified fragment length polymorphism (AFLP) analysis results in a complicated DNA fingerprint with many bands per lane relating to many different locations within the genome of an organism. Thus, AFLP analysis allows for the analysis and comparison of relatively large amounts of the genome.

All lines from crosses MC69B and MC71B that carried the Sd2 type *beta*-amylase were subjected to (AFLP) analysis to estimate the relative amounts of DNA contributed from each parent of the cross towards the genome of the lines with the Sd2 *beta*-amylase sequence (identified previously through marker-assisted selection). This technique enabled the identification of lines that had introgressed the desired thermostable *beta*-amylase, but retained more of the DNA from the agronomically better adapted Canadian parent.

AFLP analysis was carried out using the AFLP plant genome kit (Invitrogen) according to manufacturer's instructions, and the results were visualized on silver stained polyacrylamide gels.

Thirty-nine lines from cross MC69B that carried the Sd2 type *beta*-amylase were analysed with primer pair e3m3, and the results are illustrated in Figure 1. The resulting fingerprint consisted of 50-60 bands per lane. Fourteen bands (marked by an asterisk) polymorphic between the parental DNA and clearly segregating among the progeny were used in the analysis. A dendogram (Figure 2) was generated using the analysis software NTSYS. Cluster analysis identified four lines (MC69B-154, 231, 306, and 417) that were most similar to TR253, of which line MC69B-231 shared approximately 75% of its DNA (at the tested loci) with its Canadian parent. All of the lines clustered into two large groups with 25 of the 39 (64%) having more similarity to TR253.

Twenty-four lines from cross 71B were similarily analysed. The dendogram in Figure 3 was generated as before using information from 14 bands, and illustrates that few of the lines were similar to the Canadian parent line CDC Kendall. Results suggest that with the exception of three lines (MC71B-14, 41, and 69), all other lines carry a large amount of DNA from Haruna Nijo.

Lines carrying the thermostable variant of the gene, and large amounts of their genome from the Canadian parents, are preferred for their better adaptation to western Canadian growing conditions, as well as their superior malting characteristics. These lines are being evaluated further as "bridge materials" for malting-barley breeding.

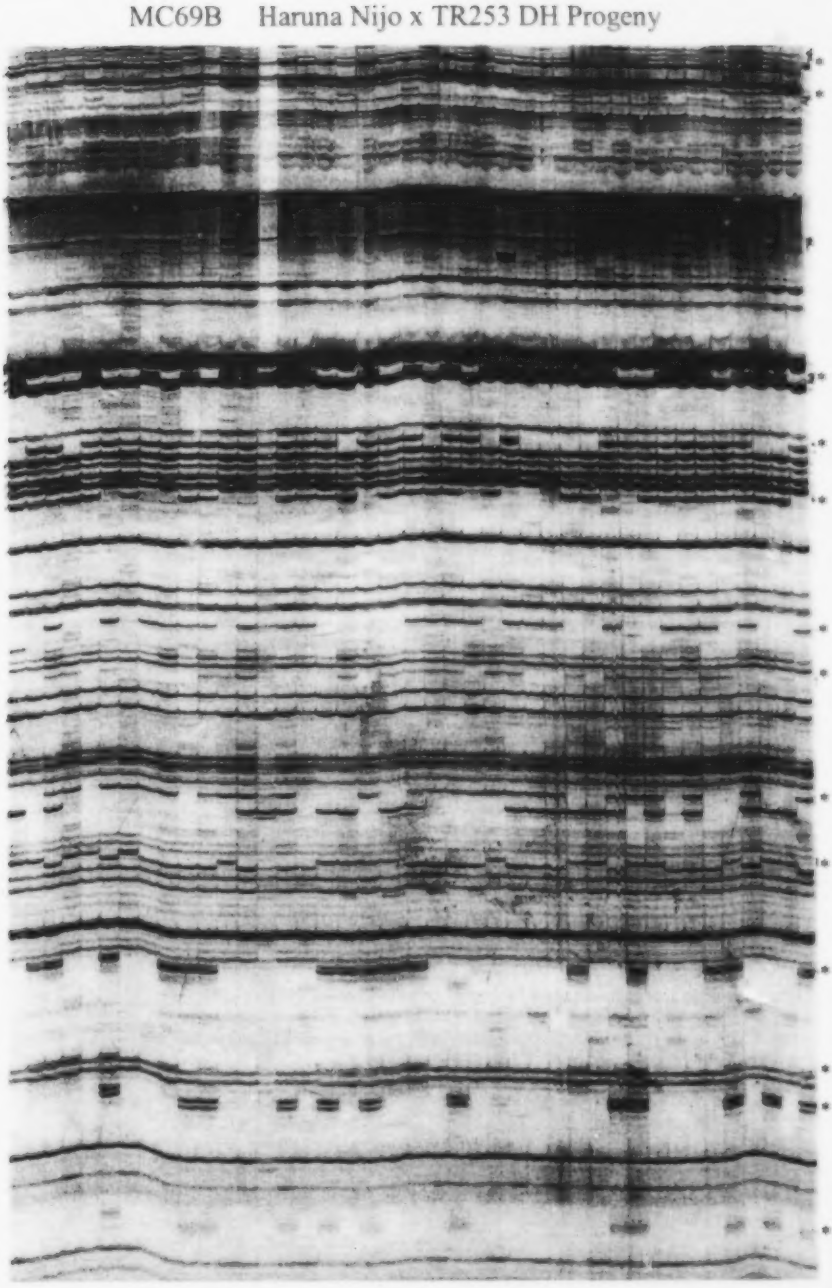


Figure 1. AFLP (primer e3m3) generated DNA banding pattern of marker-selected Sd2 DH lines from cross MC69B (Haruna Nijo x TR253). Loci (bands) that were polymorphic between the parents, and were included in the cluster analysis are indicated by an asterisk.

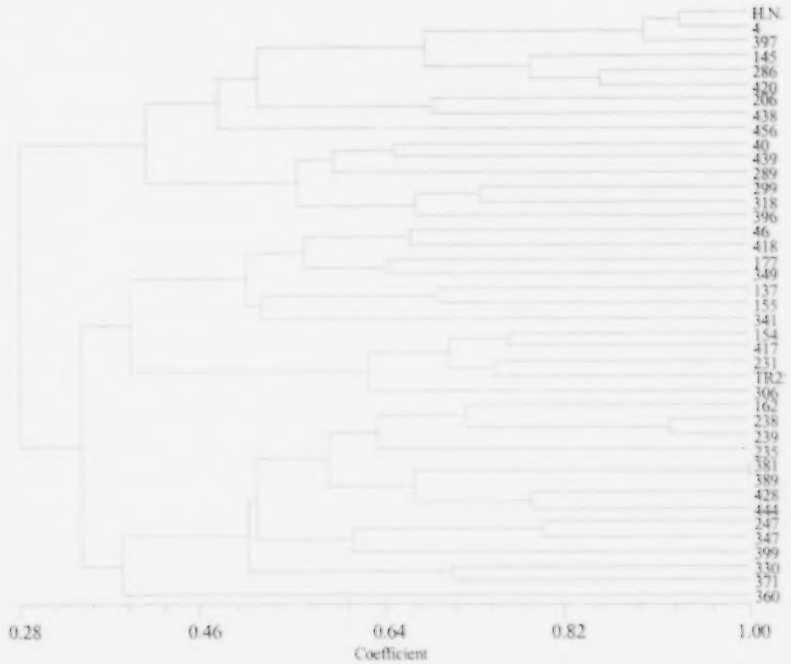


Figure 2. Cluster analysis of 39 DH lines from cross MC69B. The dendogram was generated using segregation data from 14 different loci. H.n. = Haruna Nijo, TR2 = TR253.

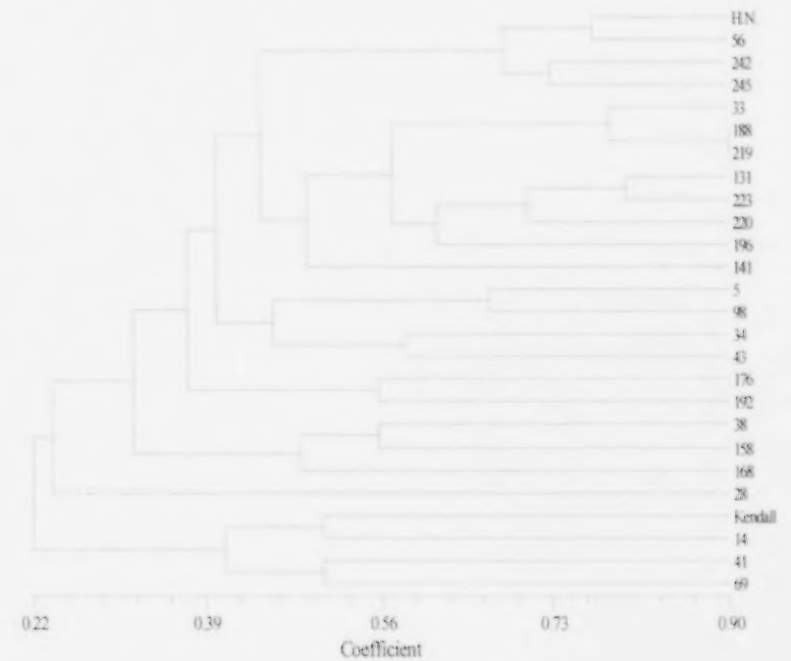


Figure 3. Cluster analysis of 24 DH lines from cross MC71B. The dendogram was generated using segregation data from 14 different loci.

Assessment of enzyme thermostability

To confirm the introgression of a functional, thermostable *beta*-amylase, the 113 lines from cross MC69B and 81 lines from cross MC71B were field grown in 2002 to obtain enough seed for *beta*-amylase analysis. The plots were grown as single 3 metre rows, under irrigation at the Preston Avenue plots in Saskatoon. Seed from the entire row was harvested for *beta*-amylase analysis.

Assays for enzyme levels were determined using a Megazyme® *beta*-amylase kit according to routinely used protocols by Dr. Michael Edney, (Grain Research Centre, Canadian Grain Commission , Winnipeg, Canada). To assess thermostability, duplicate samples of milled flour from each barley line were incubated for 15 minutes at 40°C and 60°C, and analysed for *beta*-amylase levels.

In both crosses, enzyme levels from flour incubated at 40°C varied widely, with ranges both above and below those of the parental control samples (Tables 5 and 6). Enzyme levels at 60°C were all significantly lower with a narrower range.

Table 5. *Beta*-amylase enzyme levels (Betamyl units) in marker-selected lines from cross MC69B.

Marker Indicated Group	Betamyl Units						
	40°C		60°C		% Activity Remaining		
	High	Low	High	Low	High	Low	Mean
Thermostable (Sd2)	1951.0	451.3	100.3	33.4	18.5	2.1	5.9
Normal (Sd1)	2220.8	437.0	109.8	33.4	24.1	2.0	6.2
Controls (Sd1)	1413.7	661.5	102.7	31.0	13.2	2.7	6.1

Table 6. *Beta*-amylase enzyme levels (Betamyl units) in marker-selected lines from cross MC71B.

Marker Indicated Group	Betamyl Units						
	40°C		60°C		% Activity Remaining		
	High	Low	High	Low	High	Low	Mean
Thermostable (Sd2)	1719.4	647.1	112.2	28.7	13.9	2.5	6.8
Normal (Sd1)	2232.8	434.6	133.7	28.7	14.5	2.1	6.2
Controls (Sd1)	1795.8	623.3	107.5	26.3	16.7	1.7	4.9

The percentage of activity remaining after heat treatment was lower in all samples than would be expected, and was not significantly different between the groups carrying the thermostable and normal *beta*-amylases. The distribution of residual enzyme activity among the three groups of lines was highly similar (Figure 4 A,B) and failed to verify the presence of the thermostable enzyme in the Sd2 group.

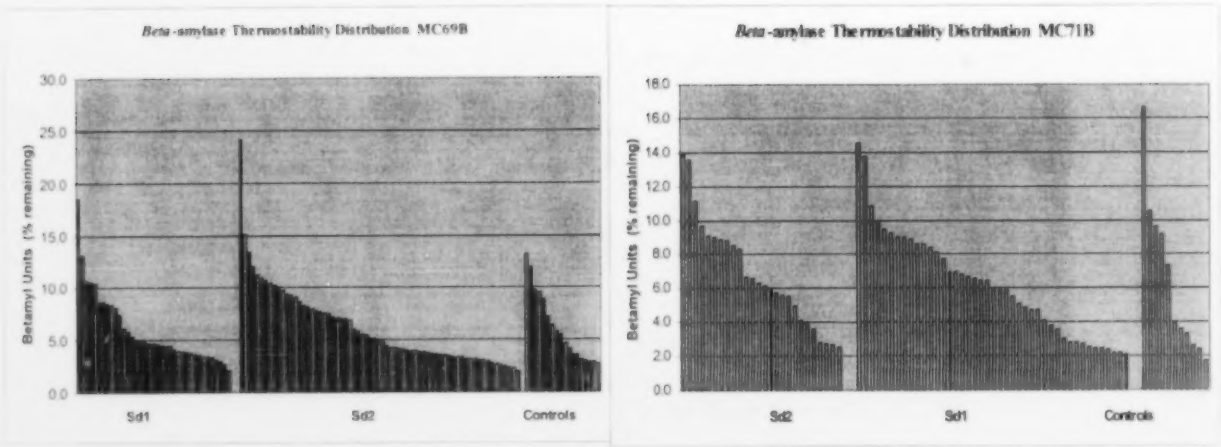


Figure 4 A,B. The distribution of residual *beta*-amylase activity according to marker-indicated Sd1 and Sd2 groups.

Expected levels of residual enzyme activity were lower than found in other studies (Eglinton et al., 1998; Kihara et al., 1998). A review of these two studies suggests that the incubation temperature, and duration at that temperature, may both have been too high, minimizing the variation that could be detected. Furthermore, the variation in enzyme activity within the controls may have been abnormal due to the poor seed lots that were available for analysis. Despite being grown under irrigation, the plots experienced one of the poorest growing seasons, and suffered from sprouting damage before harvest. Therefore we believe that our inability to properly assess *beta*-amylase activity is the main reason for the lack of confirmation of the introgression of the Sd2 type of *beta*-amylase.

Genetic diversity at the *Bmy*1 locus

All nine markers were used to examine the amount of genetic diversity present at the *Bmy*1 locus in 108 worldwide barley cultivars/lines and to assess the underlying molecular basis for Sd1 and Sd2 differentiation. The isoenzyme types were determined by a specific combination of nucleotides/amino acids present at R115, E165, V233B, A430, and 21bpI/D. Within the Sd1 subgroup, little variation existed. Only the marker BmyS347B showed any variation in Sd1 and could be used to differentiate between these genotypes. Marker BmyR115 varied in only one of the Australian lines, and all Sd1 types were similar at M527 and lack the 126bp palindromic insertion in intron III. There was considerably more variation within the Sd2 subgroup as revealed by markers BmyM527, BmyS347B, and BmyIntron3 with the specific nucleotide present at M527 and the lack of the 126bp insertion determining thermostability. Therefore, it is suggested that the amino acids arginine (position 115), aspartic acid (165), alanine (233), valine (430); in combination with the 21bp insertion, determine the Sd2 isoenzyme. In addition, the thermostable form of the enzyme requires the presence of methionine at position 527, and the lack of the 126bp palindromic insertion. Cluster analysis (Appendix C) effectively grouped all of the known Japanese Sd2-H thermostable cultivars, and this group included a line from China, and five CDC feed-barley breeding lines, including HB334 and HB345.

Since the marker BmyS347B and the microsatellite marker varied throughout, they likely do not have an important role in determining isoenzyme type. Both can however be used to differentiate between lines within the same subgroups.

Related Projects

HB334 and HB345

These lines were identified through marker analysis as carrying the same alleles in *Bmy*1 as the thermostable Japanese cultivars (Appendix C). The lines are full-sibs and have been heavily used in crosses in the CDC feed-barley breeding program, as well as crosses with several malting-barley lines. The thermostability of the *beta*-amylase in these lines had not previously been determined since the lines were feed types, thus there was no data to suggest that the lines carried a thermostable variant. The possibility therefore existed that the thermostable variant of *beta*-amylase was already present in the barley breeding program at the Crop Development Program (CDC) and that it had been crossed with several malting-barley lines currently under development. While the banding patterns of HB334, HB345, and Haruna Nijo were identical (using nine markers), the markers only tested the specific nucleotide present at several distinct points in the gene. This left the possibility that the nucleotide sequence of these lines in other regions of the gene could have been different from Haruna Nijo, resulting in an enzyme of altered thermostability. The gene was isolated from genomic DNA of HB334 and HB345, and sequenced in its entirety except for a small region on intron III, and intron VI. Sequence comparison to that of Haruna Nijo indicated that the sequences were identical in the coding (exon) regions of the gene. Four single nucleotide substitutions were found in intron III, and the microsatellite region in intron III differed in length. The genes in HB334 and HB345 are identical, and the complete similarity of the exon regions of the gene between the HB lines and Haruna Nijo suggests that the HB lines are carrying the thermostable variant of *Bmy*1. The possibility exists however that the small differences in intron III of the gene, the same region implicated in having some effect on thermostability, may alter the activity of the enzyme in the HB lines.

Preliminary results (data not shown) indicate that *beta*-amylase thermostability in the two sister lines is higher than that of typical North American cultivars. Nine crosses have been made with HB334 and HB345 (Table 7). The progeny of these crosses have been advanced to the F_6 generation. Due to previous selection for other traits, only a small number of progeny remain for each of the crosses. The remaining lines of these crosses have been screened with the markers, and based on the marker results, both Sd1 and Sd2 carrying lines will be tested for *beta*-amylase thermostability to confirm the presence of the thermostable isoenzyme type, and the ability of the markers to correctly select for the thermostable variant.

Table 7. Marker screening results for crosses involving parents HB334 and HB345 believed to be carrying the heat stable form of *beta*-amylase.

Pedigree	# of lines	Marker results
HB334 x TR640	10	6 Sd1, 4 Sd2
HB334 x Lindwall	9	3 Sd1, 6 Sd2
HB334 x Manley	44	24 Sd1, 20 Sd2
HB334 x TR251	2	2 Sd1
CDC Freedom x HB334	4	4 Sd1
HB345 x TR251	10	5 Sd1, 5 Sd2
HB345 x CDC Kendall	14	7 Sd1, 7 Sd2
HB345 x HB352	21	9 Sd1, 10 Sd2, 2 Heterozygous
HB345 x BM9216-4	8	4 Sd1, 4 Sd2

Discussion and Summary

Through the isolation and DNA sequence analysis of the *Bmy*1 gene from barley, the molecular mechanism that determines the thermostable form of *beta*-amylase has been determined. A combination of four amino acids present at specific positions within the coding region of the gene, in addition to a small insertion in the non-coding region, differentiates the two isoenzyme forms Sd1 and Sd2. Within the Sd2 subgroup, one further specific amino acid and the lack of a 126bp palindromic insertion in an intron are required for thermostability. This result confirms that of the three *beta*-amylase genes present in barley, *Bmy*1 plays an important role in *beta*-amylase isoenzyme production. This result also appears to confirm earlier speculations that elements within intron III of the gene may have a regulatory function in the determination of thermostability.

Microspore culture techniques for the rapid generation of doubled haploid (homozygous) populations at the CDC were developed during the course of this investigation, and resulted in greater breeding efficiency.

Nine molecular markers were designed and constructed to assess the levels of genetic diversity at *Bmy*1, and to efficiently select progeny carrying the desirable thermostable characteristic. The markers have been shown to be highly polymorphic illustrating their utility in a wide cross-section of the germplasm. Any of these markers can be used to track the introgression of the desirable form of *Bmy*1, as long as they are polymorphic in the parental lines in which the enzyme has been characterized. Of these markers, perhaps the most useful may be the co-dominant marker Bmy21I/D which would identify failed reactions and eliminate false negative results. The markers have been developed, are being shared with other malting-barley breeding programs, and will regularily be employed in the barley breeding programs of the CDC.

Through the use of these technologies, we have been able to introgress the Sd2 thermostable form of *Bmy*1into Canadian malting-barley breeding lines, based on molecular marker data. Our inability to phenotypically verify the introgression of the desirable gene in the progeny demonstrates the need for molecular markers to test for the actual presence of the specific form of gene. *Beta*-amylase activity is relatively simple to assess, but thermostability is often difficult to determine since the variation in mashing

temperature, while important to the malting industry, is often low. Detection of this small amount of phenotypic variation is complicated by environmental factors during the growing season, such as in 2002.
Our study identified several Canadian lines that carry a *Bmy*1 gene identical to that of Haruna Nijo. Crosses with these lines (already made) may be advantageous in that they carry the thermostable *Bmy*1 in a genetic background well suited to western Canadian growing conditions.

PERSONNEL
Molecular Techniques - Peter Eckstein (Research Officer), Cheng-Dao Li (Research Associate), Donna Hay (Technician), Akiko Tomita (Technician).
Production of doubled haploids - Gordana Radosavljevic (Technician).
Plant Crosses – Clayton Wiebe (Technician) and malting-barley field staff.
Micromalting – Doreen Frey (Technician).

EQUIPMENT
Not Applicable

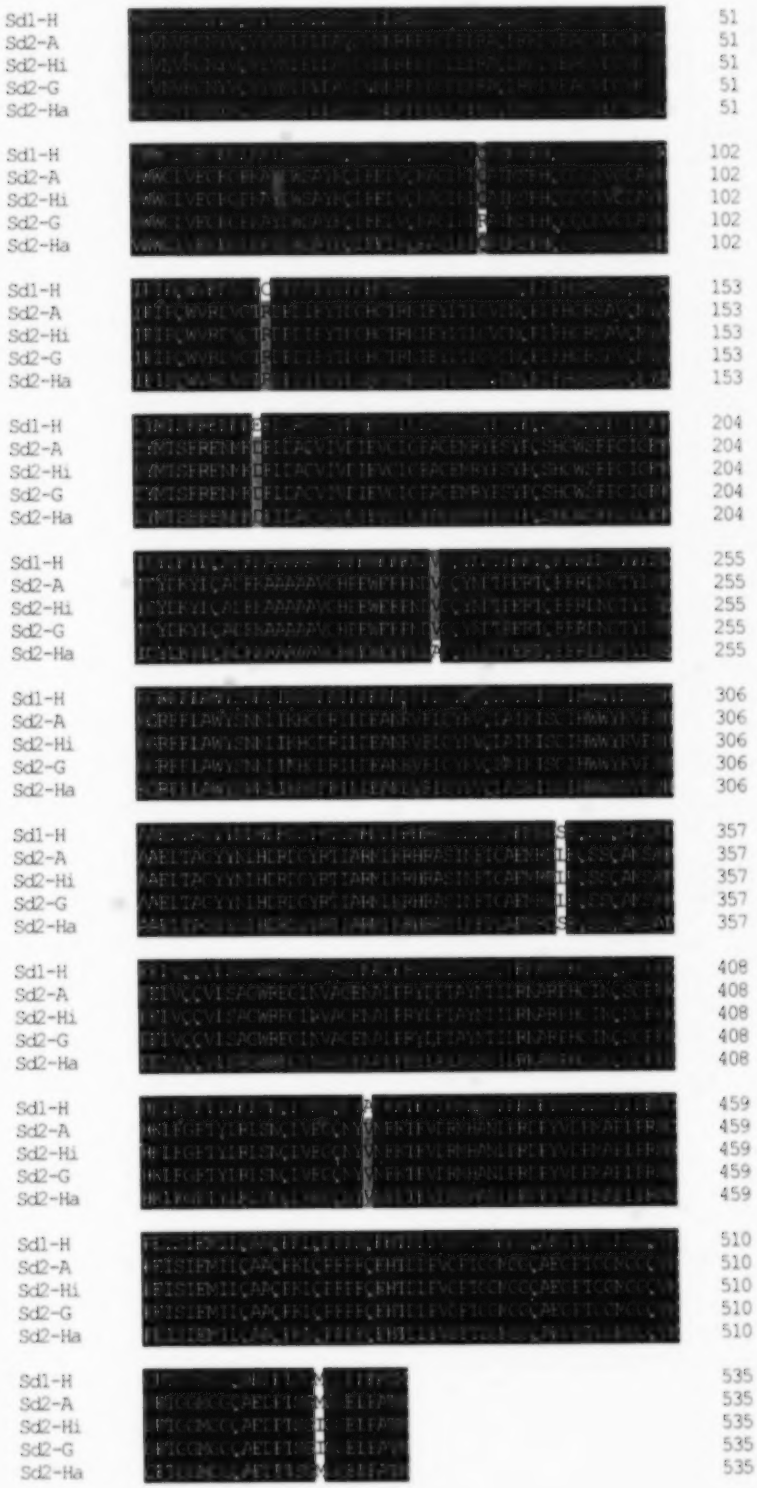
PROJECT DEVELOPED MATERIALS
All of the molecular markers, including primer sequences and reaction conditions, are freely available to other research groups.
Several barley lines have been generated through crosses with Haruna Nijo, HB334, and HB345 that are carrying the thermostable (Sd2) *beta*-amylase. These lines will serve as parental materials for future crosses, or may be registered as cultivars after several more years of field evaluations.
A manuscript reporting on the isolation and sequencing of the Sd1 *beta*-amylase from Harrington, comparison of the Sd1 and Sd2 DNA sequences and the amino acid substitution/isoenzyme type relation has been published (attached);
Li, C.-D., Langridge, P., Zhang, X.-Q., Eckstein, P.E., Rossnagel, B.G., Lance, R.C.M., Lefol, E.B., Lu, M.-Y., Harvey, B.L., and G.J. Scoles. 2002. Mapping of barley (*Hordeum vulgare* L.) *beta*-amylase alleles in which an amino acid substitution determines *beta*-amylase isoenzyme type and the level of free *beta*-amylase. Journal of Cereal Science 35:39-50.
A manuscript reporting on the genetic diversity at the *beta*-amylase locus in barley is in preparation.
Information resulting from this project has been prepared in poster format and presented at the 10th Australian Barley Technical Symposium, 2001.
Information resulting from this project is being prepared in poster format for presentation at the 9th International Barley Genetics Symposium, Brno, Czech Republic, 2004.

PROJECT PHOTOS
Not Applicable.

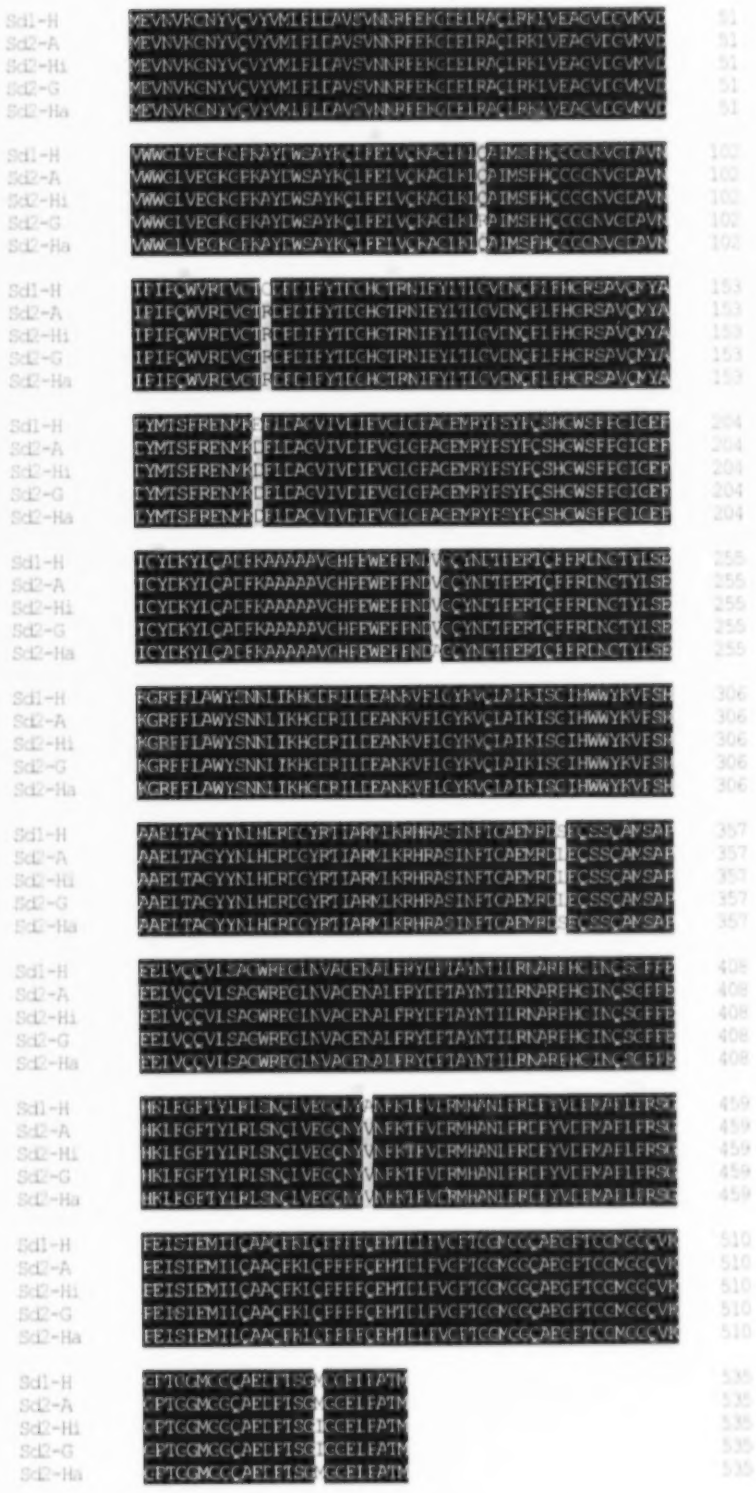
ACKNOWLEDGEMENTS
The Saskatchewan Agriculture Development Fund (ADF) is being acknowledged in all manuscripts currently published or being prepared for publication in scientific journals. In addition, we regularily acknowledge Saskatchewan ADF support during presentations and laboratory tours.

EXPENSE STATEMENT
See attached.

Appendix A. Amino acid sequence comparison of the coding regions of *Bmy*1.



Appendix A. Amino acid sequence comparison of the coding regions of *Bmv*1.



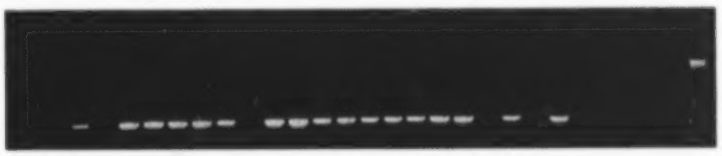
Appendix B. Banding patterns of nine molecular markers used in this study.

BmyR115. Positive signal (band) indicates the Haruna Nijo allele.

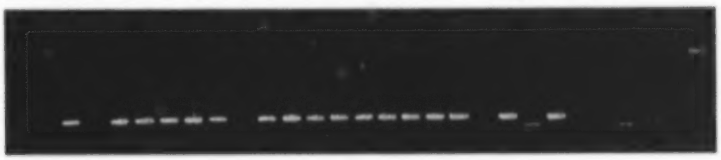
BmyS347. Negative signal (no band) indicates the Haruna Nijo allele.

BmyV233B. Positive signal indicates the Haruna Nijo allele.

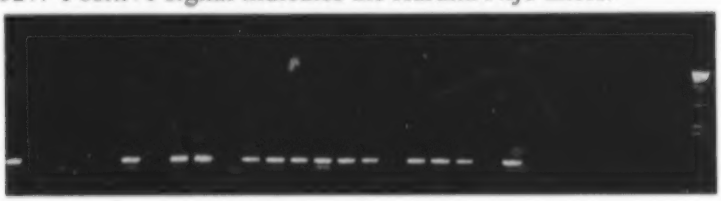
BmyE165. Negative signal indicates the Haruna Nijo allele.



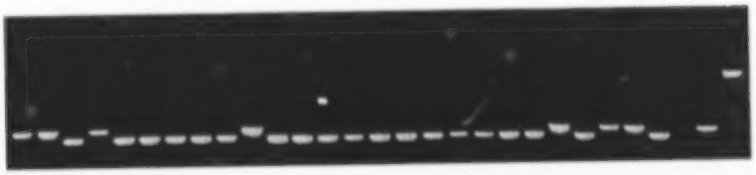
BmyA430. Negative signal indicates the Haruna Nijo allele.



BmyM527. Positive signal indicates the Haruna Nijo allele.

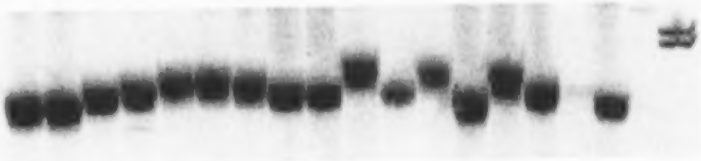


BmyIntronIII. Low band indicates the Haruna Nijo allele.

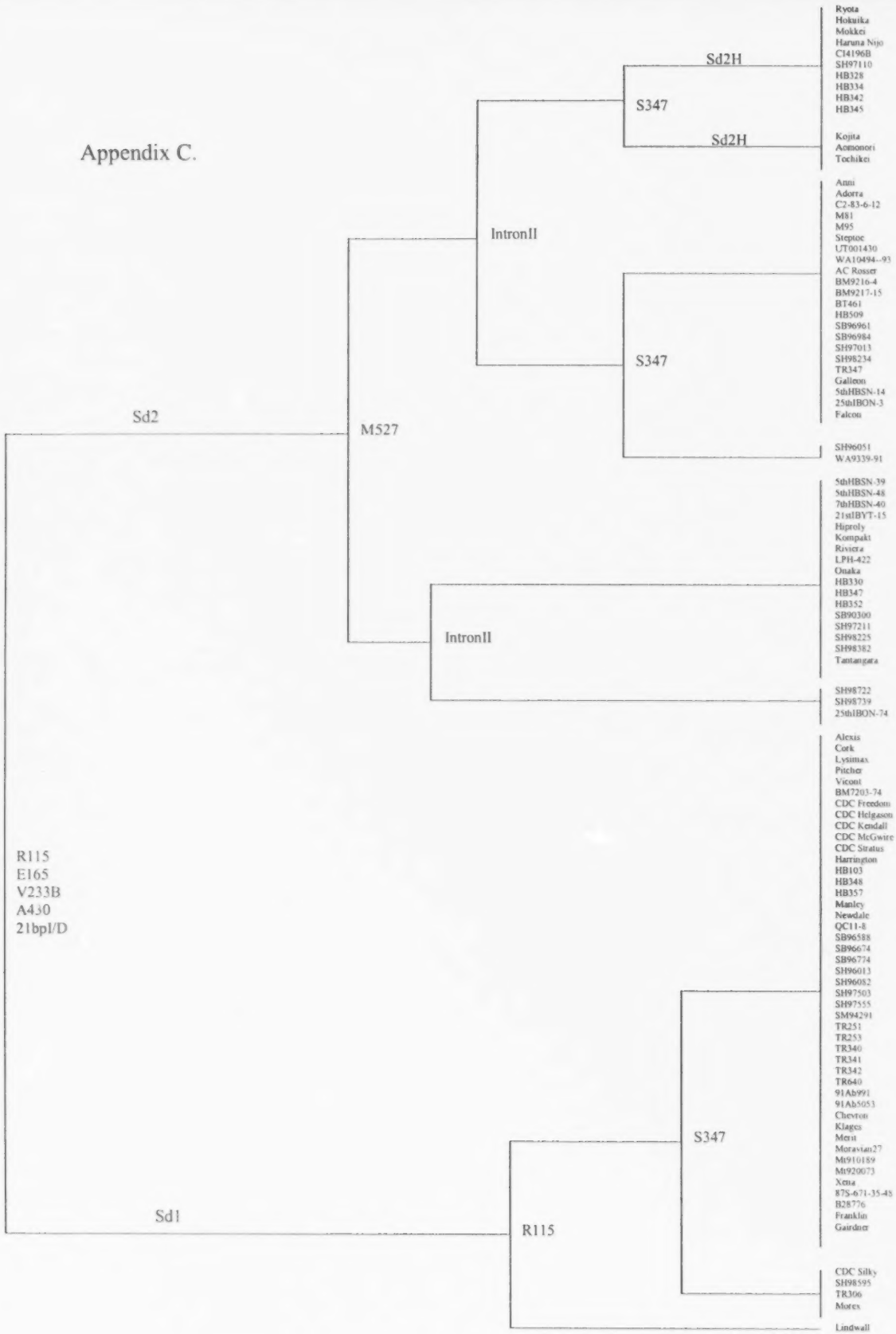


Bmy21I/D. High band indicates the Haruna Nijo allele.

Bmy Micros. Bands of different size classes identify multiple alleles.



Appendix C.


Sd2H
S347
Sd2H
IntronII
S347
Sd2
M527
IntronII
R115
E165
V233B
A430
21bpI/D
Sd1
R115
S347
Ryota
Hokuika
Mokkei
Haruna Nijo
CI4196B
SH97110
HB328
HB334
HB342
HB345
Kojita
Aomonori
Tochikei
Anni
Adorra
C2-83-6-12
M81
M95
Steptoc
UT001430
WA10494--93
AC Rosser
BM9216-4
BM9217-15
BT461
HB509
SB96961
SB96984
SH97013
SH98234
TR347
Galleon
5thHBSN-14
25thIBON-3
Falcon
SH96051
WA9339-91
5thHBSN-39
5thHBSN-48
7thHBSN-40
21stIBYT-15
Hiproly
Kompakt
Riviera
LPH-422
Onaka
HB330
HB347
HB352
SB90300
SH97211
SH98225
SH98382
Tantangara
SH98722
SH98739
25thIBON-74
Alexis
Cork
Lysimax
Pitcher
Vicont
BM7203-74
CDC Freedom
CDC Helgason
CDC Kendall
CDC McGwire
CDC Stratus
Harrington
HB103
HB348
HB357
Manley
Newdale
QC11-8
SB96588
SB96674
SB96774
SH96013
SH96082
SH97503
SH97555
SM94291
TR251
TR253
TR340
TR341
TR342
TR640
91Ab991
91Ab5053
Chevron
Klages
Merit
Moravian27
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Mt920073
Xena
87S-671-35-48
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Franklin
Gairdner
CDC Silky
SH98595
TR306
Mores
Lindwall